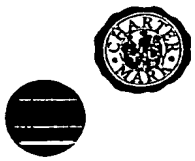


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The Patent Office
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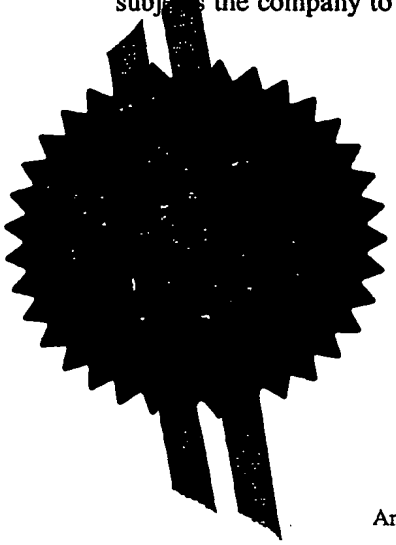

Signed Stephen Hordley

Dated 7 February 2003

31DEC01 E684710-1 D02884
P01/7700 0.00-0131026.7

1/77

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The Patent Office

Cardiff Road
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1. Your reference — P30492/CPA/RMC

2. Patent application number *(The Patent Office will fill in this part)* — 28 DEC 2001 — 0131026.7

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

   Fusion Antibodies Limited

   PO Box 374
   Belfast
   BT1 2WD

   07295982001
   Patents ADP number *(if you know it)*

   If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

4. Title of the invention — "Soluble Recombinant Protein Production"

5. Name of your agent *(if you have one)*

   "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

   Murgitroyd & Company
   Scotland House
   165-169 Scotland Street
   Glasgow G5 8PL

   Patents ADP number *(if you know it)* — 1198013 — 000011980 15

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

| Country | Priority application number *(if you know it)* | Date of filing *(day / month / year)* |
|---|---|---|
| | | |

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

| Number of earlier application | Date of filing *(day / month / year)* |
|---|---|
| | |

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*
   *a) any applicant named in part 3 is not an inventor, or*
   *b) there is an inventor who is not named as an applicant, or*
   *c) any named applicant is a corporate body.*
   *See note (d))*

   Yes

## Patents Form 1/77

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| Description | 16 |
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| Priority documents | |
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| Statement of inventorship and right to grant of a patent *(Patents Form 7/77)* | |
| Request for preliminary examination and search *(Patents Form 9/77)* | |
| Request for substantive examination *(Patents Form 10/77)* | |
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11. I/We request the grant of a patent on the basis of this application.

Signature Murgitroyd & Company Date 28/12/2001

Murgitroyd & Company

12. Name and daytime telephone number of person to contact in the United Kingdom: Roisin McNally 0141 307 8400

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**"Soluble Recombinant Protein Production"**

This application describes a methodology for the rapid production of soluble recombinant protein using high-throughput techniques. This method allows the cloning, expression and identification of soluble protein from a given target gene product by a rapid robust method. This ability to produce and analyse soluble recombinant protein in a rapid time period represents a significant advance in an area which has long been considered a significant production bottleneck in the field.

**Introduction**

The recombinant production of protein in bacteria, yeast, insect and mammalian cell lines has become a cornerstone of biological research and the biotechnology industry. Classical biochemical and chromatographical purification techniques usually produce inadequate amounts of a target protein to study its roles or actions. Even if enough of the

protein can be purified, it usually involves cumbersome amounts of starting material or tissue and many processing steps are taken before reasonable purification can be achieved.

Recombinant expression of the target protein bypasses a lot of these problems. By introducing the target protein's gene template to a cell line or bacterial culture, induced overexpression can result in significant levels of that protein being produced. Large amounts of protein make the purification a lot simpler, but the addition or fusion of purification domains or tags allows for a relatively simple one-step purification using affinity chromatography resins.

Bacteria, and more specifically, *E.coli* are ideal expression vehicles for the production of recombinant protein, as large amounts of foreign protein can be expressed in small culture volumes at low cost in comparison with other methods. However, the use of bacteria as expression hosts are not without problems. One of the most troublesome shortcomings of the use of *E.coli* is the production of the recombinant protein in an insoluble form, especially a problem when the target gene is non-bacterial. Generally, insolubility is the result of the production of protein that is not recognised by the folding enzymes, or chaperones, present in the bacterial cytoplasm. The unfolded or misfolded protein will attempt to decrease its own entropy to a minimum, and it is thought that in an effort to

hide or mask its hydrophobic residues from the aqueous environment, the protein molecules actually clump together. These clumps are insoluble and are called inclusion bodies. While in the form of inclusion bodies, the protein will have no biological activity and will be impossible to purify using affinity fusion tags. These inclusion bodies can be re-solubilised in chaotropic buffers such as 8M urea or 6M guanidine hydrochloride, but then must be dialysed against physiological buffers in an effort to refold and regain biological function. Due to the individual characteristics of each protein, this is a slow and painstaking process that may never produce active or useful protein. Therefore, the ability to quickly produce and screen soluble protein in *E.coli* represents a major step forward in protein biochemistry.

The following methodology presented describes a high-throughput process for the cloning, expression and analysis of recombinant soluble protein and protein domains. This process incorporates evaluation and comparison of many factors and conditions known to influence protein solubility at each step in order to guarantee generation of soluble recombinant protein.

According to the present invention there is provided a method of producing a soluble bioactive domain of a protein the method comprising the step of selecting suitable soluble subunits of a protein and assessing the produced protein for desired activity.

Typically the method comprises the steps of analysis of DNA coding for the protein of interest to identify antigenic soluble domains, designing oligonucleotide primers to amplify DNA encoding the domain, amplifying DNA, cloning the DNA and screening clones for correction orientation of DNA, expressing DNA in expression strains, analysing expression products for solubility, analysing products and production of soluble bioactive protein domain.

The invention is exemplified with reference to the following non limiting description and the accompanying figures.

**Methods**

**Template analysis and primer design**

The high throughput process begins with the analysis of the DNA coding for the protein of interest. Using software packages including Vector NTI (Informax, USA) and (http://www.ncbi.nlm.nih.gov/BLAST/) to identify complete domains within the protein that significantly increase the likelihood of antigenicity and solubility when expressed as a subunit of the original protein coding sequence. If possible three sub-domains are identified for processing. This has proven optimal to produce soluble protein with the majority of proteins expressed using this process.

The next step in the process is to design oligonucleotide primers to amplify these sub-domains. Primer design is aided by use of the design facility in Vector NTI the internet software package Primer3 (http://www-genome.wi.mit.edu/genome_software/other/primer3.html, (Whitehead Institute for Biomedical Research). These packages allow full control over all aspects of primer design, ranging from primer length, homology to optimal annealing temperature of the PCR reaction itself. Typically the primers designed are 20 base pairs in length with annealing temperatures of between 58-62°C and are supplied by MWG-Biotech AG (Germany).

**PCR of Insert**

The desired inserts are amplified using the primers designed specifically for that target gene. In order to maximise the chance of the PCR working without having to individually optimise each new primer set several standard conditions are used. Expand High Fidelity PCR system (Roche, Basal, Switzerland), is used for the reaction, working stocks of each primer are made (10pMol/µl) and each PCR is set up to be carried out under 3 different thermocycler conditions in every case.

1. A standard PCR programme using the recommended annealing temperature provided with the primers.
2. A standard PCR programme using 50°C as the temperature for annealing.

3. A touchdown PCR programme, where the annealing temperature starts at 65°C for 10 cycles and then gradually decreasing the annealing temperature to 50°C over the following 15 cycles.

The PCR products are then visualised on a 1.5% agarose gel stained with Ethidium Bromide (example in Figure 1) and the bands are cut out of the gel and purified using Mini elute gel extraction Kit (Qiagen, Crawley, England).

**Colony Screening for Inserts in Correct Orientation**

Ten of the transformants are screened using directional PCR using a primer that encodes for a sequence on the vector such as S Tag or GATA sequence, and then the complementary primer from the insert. A PCR mix is used - RedTaq DNA Polymerase (Sigma Aldrich, Dorset, England) and the thermocycler conditions used are the standard PCR programme using 50°C as the annealing temperature.

The PCR products are visualised as shown in figure 1. Positive clones are then inoculated in a small-scale culture (750µl) and grown at 37°C for 5 hours with shaking and then the plasmid DNA is extracted using SpinPrep 20 Plasmid Kit (Novagen, Madison, USA). The sequence of these clones is checked using sequencing.

We do not wish on most occasions to express the whole region of a target protein as this is only likely to cause insolubility problems, and it also presents less problems for our GMAG submissions. Therefore using bioinformatics software we intend to identify globular regions or domains of the target protein (if not already known) and amplify these regions.

Selection of clones and generation of constructs is shown in Figures 2 and 3.

**Sequencing**

The sequencing reactions are performed on the cloned inserts, using the Big Dye Terminator cycle sequencing kits (Applied Biosystems, Warrington, UK) and the specific sequencing primer run on a Peltier Thermal cycler model PTC225 (MJ Research Cambridge, Mass). The reactions are run on Applied Biosystems - Hitachi 3310 Sequencer according to the manufacturer's instructions. These sequences are checked to ensure that no PCR generated errors have occurred.

**High Throughput Screening of Expression Clones**

**1. Growth and Expression of Clones**

For each construct four plasmids are selected and subcloned into *E. coli* strains (Rosetta(DE3)pLacI, Tuner(DE3)pLacI, Origami B(DE3)LacI). Following re-

transformation into the 3 *E. coli* expression strains, 4 colonies from each are chosen for high throughput screening. The ability to quickly screen clones for the production of the recombinant protein is absolute key to the successfulness of the present Application high-throughput system.

Basic Setup of Protocol

1. Identify four positive clones on the NovaBlue transformation plates and miniprep and sequence.

2. Use these 4 minipreps to transform the three chosen expression strains. These are
   a. Rosetta(DE3)pLacI
   b. Tuner(DE3)pLacI
   c. Origami B(DE3)LacI

3. This will generate 12 agar selection plates for each insert being studied. Take 4 colonies per plate and use it to inoculate in duplicate two wells in the 96 well plate and grow this as the primary culture. The setup of the plates is as follows is describe in the flow diagram (Figure 8).

The colonies are used to inoculate duplicate wells in a 96 well plate. Each well contained 200 µl of LB broth with the appropriate antibiotics. Each plate is dedicated to one strain of *E. coli* which alleviates the problems of different growth rates. The necessary controls are also included on each plate. The plates are then grown up at 37°C, with

shaking, until log phase is reached. This is the primary plate. From the primary plate a secondary plate is seeded using 'hedgehog' replicators. This, secondary, plate is then grown up to log phase, chilled to 16°C for 1 hour then induced with IPTG, to a final concentration of 1 mM. The plates are then grown up at 16°C overnight. The primary plate is stored at 4°C until the process is complete.

**2. Harvesting and Lysis Of Cells**

The contents of each well in the 96 well plate are transferred into a Millipore 0.65 µm multi-screen plate. The plate is placed on a manifold and a vacuum is applied. This draws off the culture medium to waste. The cells are then be washed with PBS (optional), again the vacuum is applied to remove the PBS. The multi-screen plate is removed from the manifold and bacterial cell lysis buffer (containing DNAse) (50 µl) is added to each well. The plate is incubated at room temperature for 30 minutes with shaking to facilitate lysis of the cells. A fresh 96 well microtitre plate (ELISA grade) is placed inside the vacuum manifold and the multi-screen plate is placed above it. When a vacuum is applied the contents of each well are drawn into the micro-titre plate below. The vacuum only needs to be applied for 20 seconds. The collected lysate contains the soluble fraction of expressed protein. To analyse the insoluble fraction, urea buffer (50 µl) is added to the wells of the multi-screen plate and it is incubated at

room temperature for 30 minutes with shaking. The contents of each well are removed manually using a multi-channel pipette and are either stored or used directly in a dot blot (see next stage).

3. Analysis of the Soluble and Insoluble Fractions

The soluble fraction of the cell lysate are analysed either by direct ELISA or by dot blot:

ELISA : 2X sodium carbonate buffer (50 µl) is added to each well and the plate is incubated at 37°C for 1 hour. The plate is inverted to remove the contents and subsequently blocked with 3% BSA in TBS-T (300 µl/well). The plate is incubated at 37°C for 1 hour. The blocking reagent is removed as before and the wells are probed with anti-polyHis-AP antibody for 30 minutes at 37°C. The plate is washed three times over 10 minutes with TBS-T and substrate is added to each well to disclose soluble expressed protein.

Dot Blot: Dot blot analysis can be used for both soluble and insoluble fractions. The samples are applied to the dot blot apparatus housing nitrocellulose membrane. The insoluble fraction dot blots are washed with TBS (200 µl) to remove excess urea. The membranes are blocked for 30 minutes at room temperature (3% BSA in TBS) then probed with anti-polyHis-AP antibody for 30 minutes at room temperature. The blots are then washed once with TBS, 5 minutes at room temperature, then once with

alkaline phosphatase buffer, 5 minutes at room temperature. Positive clones are then identified following the addition of alkaline phosphates substrate.

**Analysis of Products**

Four proteins (arbitrarily named A, B and C) are analysed using the above method. Colonies harbouring plasmids expressing each of the 4 proteins are grown up in a 96 well plate and harvested as described. Soluble and insoluble fractions are prepared and analysed both by dot and Western blot.

**Isolation of Insoluble Expressed Protein**

Analysis of the insoluble material provides a complete picture of the expression of recombinant protein. To facilitate analysis of the insoluble fraction, urea buffer (50 µl) was added to each of the wells of the multi-screen plate. The plate was incubated at room temperature for 30 minutes with shaking. The contents of each well were then removed manually using a multi-channel pipette and were either stored or used directly in a dot blot (as described).

Of the 4 proteins investigated one, namely protein A, is found to be expressed in a soluble form as confirmed by dot blot of the soluble cell lysate fraction. Soluble product is not observed with the

other proteins investigated as confirmed by dot blot analysis of both the soluble and insoluble fractions (refer to figures 4 and 5).

These results are further confirmed by Western blot analysis of the expression from three colonies for each of the proteins (refer to figure 6).

The primer design requires the upstream primer to contain an ATG start codon and the downstream primer to contain the antisense for a stop codon. We have added our own requirements to this primer design to meet with the high-throughput requirements of the present Application system. These requirements are,

A. In the upstream primer, the start ATG codon should be preceded with a G or a A. This simple addition means that the same forward primer can be used for the insertion of an insert into all vectors (the G fulfils a AAG Lys codon (or AAA if another A used) at the end of the enterkinase site in the GST tag).

B. The stop codon to be used is TAA, therefore the reverse primer should start with the sequence ATTA, with the additional T aiding the efficiency of the stop codon.

C. Incorporation of histags is achieved by the addition of the coding region in the primer, forward or reverse as required.

D. The addition of a TAG stop codon is essential in the native reverse primer. This is to act as a stop codon when cloned in the opposite direction as to what is required when using the vector. When cloning into the vector, an overexpressed protein will be produced matter what orientation the insert is in (due to the expression of the GST tag). On gels and blots it would be very hard to tell a positive clone from a negative if the target insert is very small (due to the translation of rubbish from the insert in the wrong orientation). The addition of this TAG in the reverse primer means that when the insert is in the wrong orientation, then no translation beyond the GST tag will occur. This should make positive identification of positive expression much easier on gels and blots.

1. Gradient PCR. The use of the 96 well block allows the use of an optional annealing gradient. Therefore, we can tailor the block to suit the calculated annealing temperature for each reaction

2. Touchdown PCR. This starts at a annealing temperature likely to be above the true value and decreases slowly with cycles, so that can increase the chance of amplifying the product if it is likely to be problematic.

3. 50°C annealing temperature. This is a program of last resort. It is likely to produce more than one band, but can be invaluable with problem inserts

Following PCR, the products are then analysed by agarose electrophoresis and also purified by gel excision as this minimizes the chance of carrying template plasmid clones through into the cloning procedures, which may result in false positives on the transformation selection plates.

Positive clones can then be propagated to perform a plasmid miniprep, which subsequently can be used for DNA sequencing and transformation into proper expression strains. It is possible to induce expression in NovaBlue cells, but this is a slow growing strain that it not protease deficient unlike some of the tailormade B strains that are available on the market.

**High Throughput Expression Evaluation**

The ability to quickly screen clones for the production of the recombinant protein is absolute key to the successfulness of the whole present Application system. We select 4 clones from each cloning transformation, and after miniprep'ing, to analyse 4 colonies in duplicate per plate.

1. This format is performed as each plate is dedicated to one strain, as to mix strains on the one plate is awkward due to the different growth rates. Therefore, each plate can be made up for the analysis of 2 constructs. This initial plate is called the primary plate and is grown at 37 °C until the cultures reach late log phase

2. From this plate, secondary plates are seeded using 'hedgehog' replicators, and these are again grown up to log phase, then induced with IPTG and left to grow overnight at 25°C

**Dot-Blot Analysis of Expression**

Once we have induced and grown the cultures, the presence of recombinant protein, and its solubility is determined. This is performed using dot-blots, then screening the dot blots with anti-histag antibody

1. Firstly, the grown plate is spun at 4K g to pellet the bacterial cells and the supernatant is simply removed by inversion of the plate

2. The cell pellet is resuspended in bacterial lysis buffer and after lysis, spun again to separate soluble and insoluble protein

3. A portion of the supernatant (soluble fraction) is taken and applied to a dot blot for total analysis of soluble protein

4. Resuspended pellet is then applied to a second dot blot for the analysis of insoluble protein for each of the clones

This analysis provides a picture of the expression status of the clones on each plate. Using these we then select positive soluble protein expressing

clones and scale their growth up to 5 ml scale, using the saved primary plate as an inoculum.

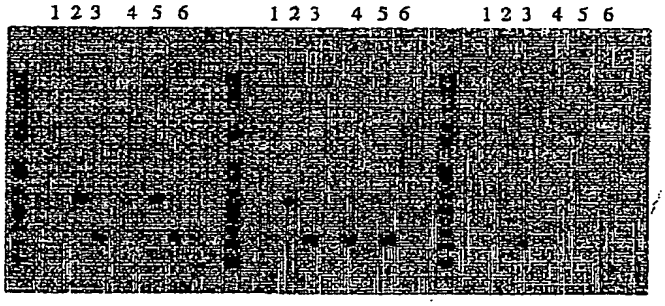
These will be induced and grown in a similar manner for the preparation of a small scale NiNTA prep to identify whether or not the soluble protein will bind to NiNTA, thus facilitating purification. This can be checked by SDS-PAGE and western blotting of the samples pre and post purification with the NiNTA columns. At this point have identified good candidates for large scale expression and purification.

Figure 7 shows a putative timetable for the process from the time that the order is placed to expression of immunisation-ready protein.

**Figure 1**

PCR insert gel.
Labelled 123 456

1=Fragment 1 minus DMSO
2=Fragment 2 minus DMSO
3=Fragment 3 minus DMSO
4=Fragment 1 plus DMSO
5=Fragment 2 plus DMSO
6=Fragment 3 plus DMSO




(6 bands - each set are separate by molecular weight markers), Set 1 are for the 50°C reaction, set 2 are the actual annealing temp for primers, and set 3 are the touchdown PCR.

**Figure 2**

Colony PCR gel
Labelled 1-10. these labels correspond to the colonies screened ie: 10 from each plate. +=Positive control
-=negative control.

first 1-10 are colonies from fragment 1

The second set labelled 1-10 are colonies from fragment 3

The third set labelled 1-10 are colonies from fragment 2.

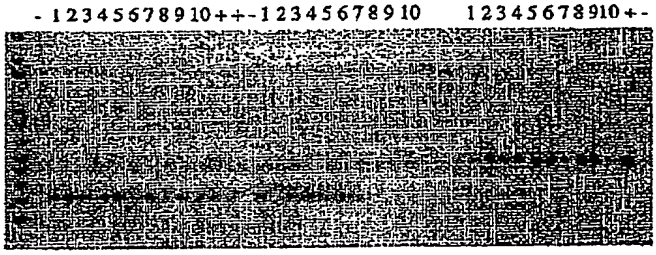


**Figure 3**

**These constructs are generated:**

1. 

N-teminal Histag target protein

2. 

target protein C-terminal Histag

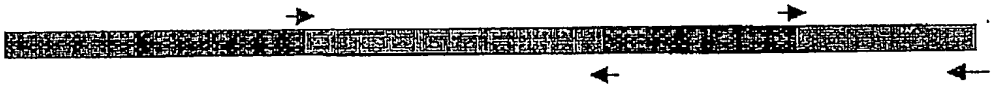
3. 

GST tag Histag target protein

Primer design using guidelines in the previous section can be facilitated quickly and easily using Vector NTI and Primer3 software.

In the situation described below, where a protein contains 2 globular domains, we could amplify some 9 constructs, as demonstrated below. However, we chose the 4 most appropriate.

(globular regions are shown in light grey)

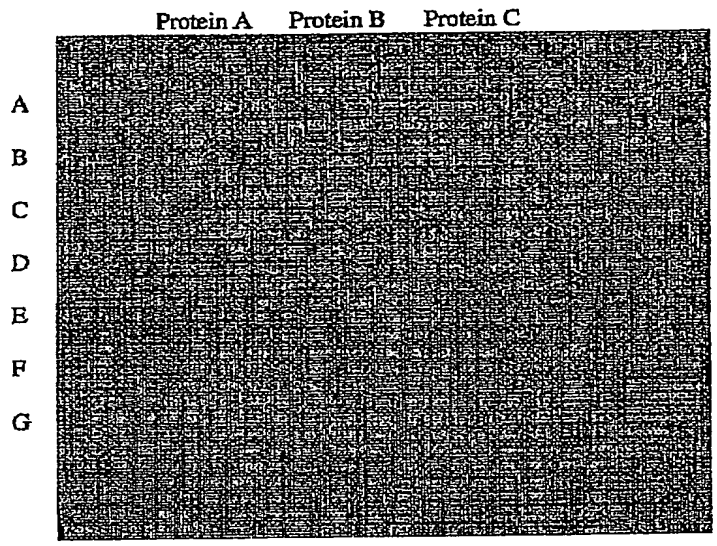


The arrows demonstrate the points at which we wish the primers to anneal to the template cDNA. (draw 4 possible products)

Using our requirements for the design of the primers, we can effectively produce 4 different inserts using only 4 primers, as opposed to the many more it would require based on the strategies for each of the vectors on their own.

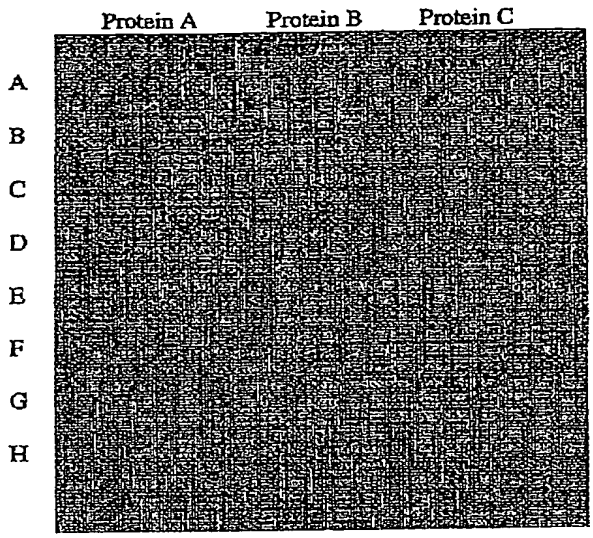
The inserts shown on the diagram can be cloned into any vector with the use of a TA cloning kit, the use of a high fidelity *Taq* is required, so that A overhangs are left on the PCR products. The other three native fragments are specially designed to be inserted into the vector, which requires the use of blunt ended products, which can be produced when using a proof-reading polymerase such as *Pwo* or *Pfu*.

Figure 4 (Dot blot analysis of soluble fractions)



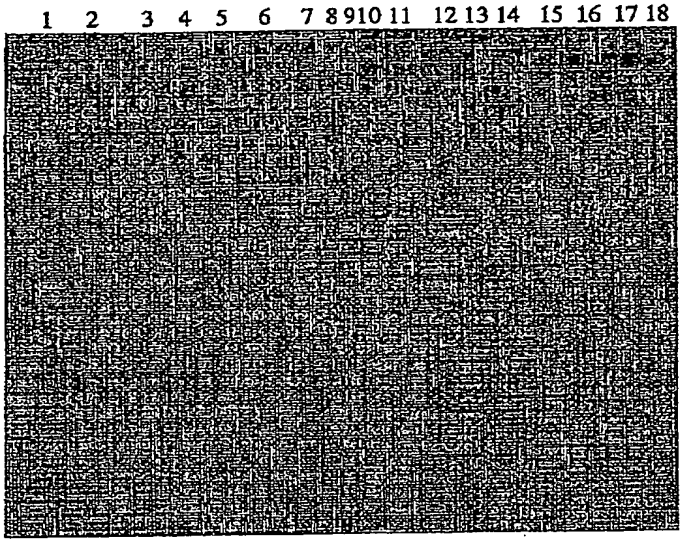

Rows A-F represent duplicates of 6 clones
Row G –lysis buffer only

Figure 5 (Dot blot analysis of insolnble fractions)




Rows A-F represent duplicates of 6 clones
Row G lysis buffer only
Row H urea buffer

Figure 6 (Western blot analysis)




Lane 1 markers
Lane 2 soluble protein A (clone 1)
Lane 3 insoluble protein A (clone1)
Lane 4 soluble protein A (clone 2)
Lane 5 insoluble protein A (clone 2)
Lane 6 soluble protein A (clone 3)
Lane 7 insoluble protein A (clone3)
Lane 8 soluble protein B (clone 1)
Lane 9 insoluble protein B (clone1)
Lane 10 soluble protein B (clone 2)
Lane 11 insoluble protein B (clone 2)
Lane 12 soluble protein B (clone 3)
Lane 13 insoluble protein B (clone 3)
Lane 14 soluble protein C (clone 1)
Lane 15 insoluble protein C (clone1)
Lane 16 soluble protein C (clone 2)
Lane 17 insoluble protein C (clone2)
Lane 18 soluble protein C (clone 3)
Lane 19 insoluble protein C (clone3)

Figure 7

**Timetable for Production of Protein**

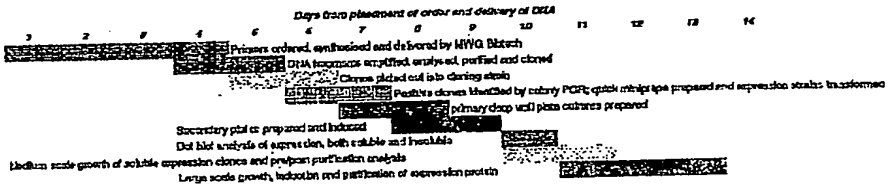
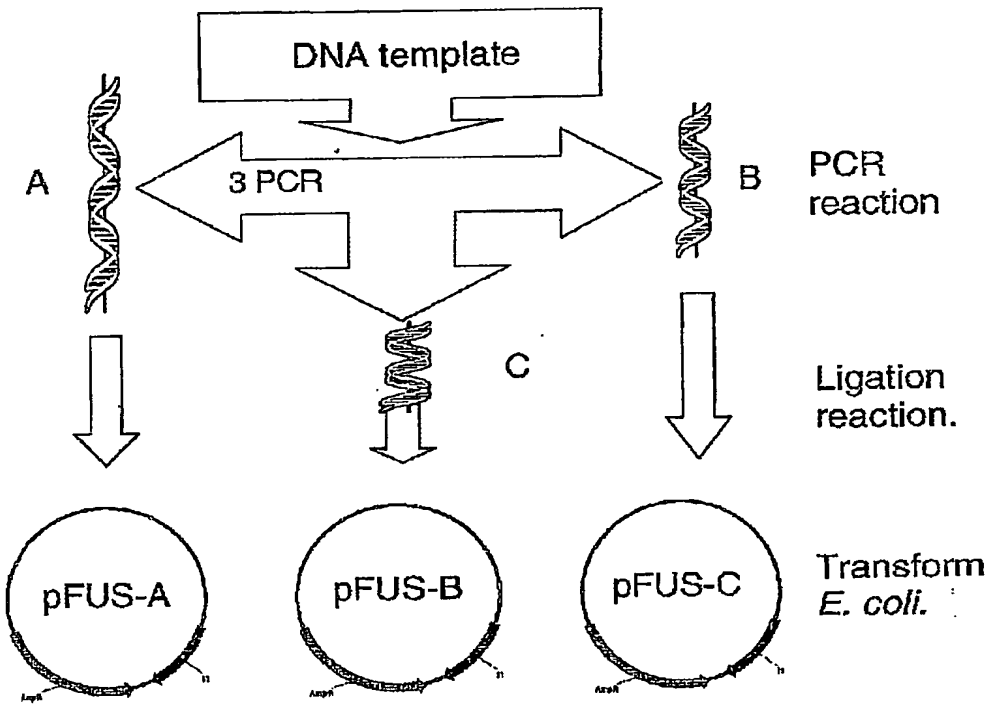
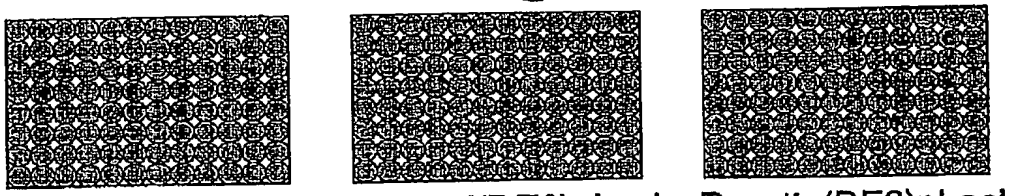


Figure 8




4 clones from the first transformation experiment are selected and used to transform 3 *E. coli* expression strains. 4 colonies from each expression strain are screened in duplicate for soluble proteins, resulting in 3 x 96 clones screened from each template.



Tuner(DE3)pLacI

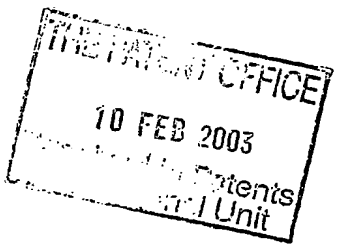
Origami(DE3)pLacI expression cells

Rosetta(DE3)pLacI expression cells

Flow chart of the fusion antibodies high-throughput process

PCT/GB2002/005941

P. T.



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